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13. ABSTRACT (Maximum 200 words)

This project tested the hypothesis that Gly-Gin, a dipeptide synthesized from the opioid peptide beta-endorphin inhibits hemorraghic hypotension in laboratory animals and attempted to determine the physiological and receptor mechanisms of its action. Tests in laboratory animals indicated that Gly-Gin produced a dose-related inhibition of hemorraghic hypotension when injected immediately before blood withdrawal. It also elevated plasma norepinephrine suggesting that it raises arterial blood pressure in hemorraged rats by restoring sympathetic nerve activity. Thus, Gly-Gin is a potent antagonist of opioid and hemorraghic hypotension. This project will be continued at the PI's new location at the Albany College of Pharmacy in Albany, NY. Also included herein are the Annual Report Questionaire and Project Highlight for the period 1Jun98 to 31May99.

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UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

Final Technical Report

GRANT TITLE: Control of Hemorrhagic Hypotension with Gly-Gln, a Non-Opioid ß-Endorphin Peptide

PRINCIPAL INVESTIGATOR: William R. Millington (email:wmillington@cctr.umkc.edu

INSTITUTION: University of Missouri-Kansas City

GRANT NUMBER: N00014-98-1-0249

AWARD PERIOD: 1 Jan 1998 – 30 Sep 1999

REPORTING PERIOD: 1 January, 1998 - 30 Sep 1999

OBJECTIVE: To test the hypothesis that Gly-Gln, a dipeptide synthesized from the opioid peptide, ß-endorphin, inhibits hemorrhagic hypotension in laboratory animals and to determine its physiological and receptor mechanisms of action.

APPROACH: This research will test two specific hypotheses regarding Gly-Gln's mechanism of action. First, it test the hypothesis that Gly-Gln prevents hemorrhagic hypotension by activating sympathetic neurons. This objective will be met by monitoring renal sympathetic nerve activity, peripheral vascular resistance and plasma catecholamine concentrations following intraventricular or site specific Gly-Gln administration to rats. Secondly, it will test the hypothesis that Gly-Gln's central actions are mediated by a specific, Gly-Gln receptor. Gly-Gln receptors will be characterized with a radioligand binding assay using [^{3}H]Gly-Gln as a ligand and a functional calcium uptake assay using multiimaging video microscopy.

ACCOMPLISHMENTS (last 12 months):

A. Background: Opioid neurons are thought to play an important role in hemorrhagic shock. Hemorrhage produces a biphasic response. Initially, arterial pressure is maintained by a compensatory increase in vascular resistance and heart rate. Following severe blood loss, a second phase develops in which sympathetic activity suddenly decreases and arterial pressure falls precipitously. The second, sympathoinhibitory phase of hemorrhage can be prevented with naloxone or other opioid receptor antagonists suggesting that opioid peptide neurons trigger hemorrhagic hypotension. Naloxone is often contraindicated by the need for opioid analgesics, however, and its therapeutic utility remains controversial.

To circumvent the liabilities of opioid antagonists, we focused our research on the biosynthesis of opioid peptides, specifically ß-endorphin. The potent opioid properties of ß-endorphin are widely recognized, but certain ß-endorphin neurons convert ß-endorphin$_{1-31}$ to non-opioid derivatives: ß-endorphin$_{1-27}$, ß-endorphin$_{1-26}$ and ß-End$_{30-31}$, or glycyl-glutamine (Gly-Gln). We became interested in the pharmacology of Gly-Gln following reports that it inhibits the firing frequency of neurons in brainstem regions that govern cardiovascular and respiratory function and that Gly-Gln is synthesized from ß-endorphin in the brainstem, but not in forebrain sites where ß-endorphin participates in pain control. Thus, we hypothesized that Gly-Gln may prevent hemorrhagic or opioid hypotension without affecting opioid analgesia or pain perception.

Initially, we investigated whether Gly-Gln administration to rats influences arterial pressure or heart rate. Intraventricular (icv) Gly-Gln injection potently inhibited the hypotension produced by ß-endorphin or morphine administration although it did not change arterial pressure in normotensive animals. These findings prompted us to test whether Gly-Gln inhibits hemorrhagic hypotension. Gly-Gln produced a

dose-related inhibition of hemorrhagic hypotension in conscious rats when injected immediately before blood withdrawal. Gly-Gln also elevated plasma norepinephrine suggesting that it raises arterial pressure in hemorrhaged rats by restoring sympathetic nerve activity. Thus, Gly-Gln is a potent antagonist of opioid and hemorrhagic hypotension.

B. Effect of Gly-Gln on Morphine Analgesia and Respiratory Depression: Although these earlier studies showed that Gly-Gln prevents opioid and hemorrhagic hypotension, they do not indicate whether Gly-Gln differs from classical opioid receptor antagonists. During the current project period, we completed preliminary investigations of Gly-Gln's effect on morphine-induced respiratory depression and analgesia. Respiratory depression is morphine's most serious side effect, particularly for trauma patients. It is often treated with naloxone, but naloxone also blocks opioid analgesia. Hence, there is a need for treatments that prevent opioid-induced respiratory depression but do not compromise opioid analgesia.

The hypothesis that Gly-Gln inhibits morphine-induced respiratory depression was investigated by monitoring plasma pCO_2, O_2 and pH in conscious rats (in collaboration with Dr. Medge Owen at Wake Forest School of Medicine). Gly-Gln (1, 3, 10, 30 or 100 nmol) or saline (5 µl) was injected icv followed, 4 min thereafter, by icv morphine (40 nmol) or saline; arterial blood (0.4 ml) was withdrawn immediately before and 35 min after Gly-Gln administration. Morphine injection produced significant respiratory depression; pCO_2 rose from 34.8 ± 1.0 mm Hg to 52.3 ± 2.7 mm Hg and pO_2 fell from 86.4 ± 1.7 to 54.1 ± 1.9 within 30 min. Correspondingly, plasma pH declined from 7.47 ± 0.01 to 7.30 ± 0.01. Pretreatment with Gly-Gln (1-100 nmol) produced a dose-related attenuation of morphine-induced hypercapnia, hypoxia and acidosis; the lowest dose to produce a significant inhibitory response was 1 nmol. When given alone to rats that had not received morphine, Gly-Gln (1, 10 or 100 nmol) had no effect on pCO_2, pO_2 or pH indicating that it does not act as a respiratory stimulant independent of the effects of morphine. Thus Gly-Gln inhibits morphine-induced respiratory depression in conscious rats without influencing respiratory function in otherwise normal animals.

In parallel experiments, we tested whether Gly-Gln inhibits morphine antinociception at doses necessary to inhibit respiratory depression. To test this, we used the paw-lift test, which measures the time duration necessary for a freely moving rat to remove its paw from a thermal stimulus. Gly-Gln had no effect whatsoever on morphine analgesia. Morphine (30 nmol icv) increased response latencies to 46.2 ± 11.4 % maximum possible effect (MPE) within 10 min; response latencies were not significantly different when Gly-Gln (1 - 300 nmol) was given concurrently with morphine. Gly-Gln (1-300 nmol) also failed to affect ß-endorphin (0.5 nmol) induced antinociception in the tail flick reflex test and did not influence paw lift or tail flick latencies when given alone to rats that did not receive morphine or ß-endorphin. Thus, Gly-Gln does not affect opioid analgesia or pain perception when given icv at the same or higher doses than those required to inhibit morphine-induced respiratory or cardiovascular depression.

C. [^{3}H]Gly-Gln Binding Sites: A primary objective of this research project, is to discover Gly-Gln's receptor mechanism of action. During the current project period, we investigated Gly-Gln receptors by using a radioreceptor binding assay with [^{3}H]Gly-Gln (5 or 49 Ci/mmol) as the radioligand. [^{3}H]Gly-Gln binding to bovine brain membranes fulfilled many of the criteria for a neurotransmitter receptor. [^{3}H]Gly-Gln binding was saturable, with a K_d = 44 ± 9 nM and Bmax = 1.5 ± 0.3 pmol/mg protein, linear with time and protein concentration and temperature and pH dependent. Furthermore, [^{3}H]Gly-Gln binding sites were localized subcellularly in synaptosomes and found regionally in highest density in the pons and medulla. [^{3}H]Gly-Gln apparently binds to a unique, Gly-Gln binding site, rather than a previously identified receptor, because it failed to displace radioligands for opioid, glycine or a variety of other neurotransmitter receptors. Conversely, [^{3}H]Gly-Gln binding was not displaced by ligands for glycine, opioid, excitatory amino acid or other known receptors. Initial structure activity studies demonstrated that [^{3}H]Gly-Gln is displaced by dipeptides structurally related to Gly-Gln although relatively minor modifications substantially reduced affinity. Hence, based on binding studies alone, the characteristics of [^{3}H]Gly-Gln binding are consistent with those of a neurotransmitter receptor.

SIGNIFICANCE: Opiates are the mainstay of pain therapy but they can be detrimental to severely injured patients. The finding that Gly-Gln inhibits hemorrhagic and opioid hypotension without affecting morphine analgesia suggests that Gly-Gln analogs may be useful adjuncts for treating hemorrhagic shock, particularly when life support technologies are not immediately available or when opiates may potentiate hemorrhagic hypotension.

WORK PLAN (next 12 months):

1. Investigate Gly-Gln's physiologic mechanism of action. These experiments will test whether Gly-Gln prevents hemorrhagic hypotension by activating sympathetic neurons. Toward this end, we recently initiated experiments to determine if Gly-Gln stimulates renal sympathetic nerve activity, induces tyrosine hydroxylase activity in sympathetic ganglia, or raises plasma catecholamine and/or vasopressin concentrations.

2. Determine Gly-Gln's site of action. Initially, these experiments will test the hypothesis that Gly-Gln acts within the periaqueductal gray region (PAG). This hypothesis is based on evidence that PAG neurons are activated by trauma and severe hemorrhage. Furthermore, the PAG is densely innervated by ß-endorphin neurons and is thought to the site where ß-endorphin produces analgesia during extreme stress and trauma. Recently, we found, in preliminary experiments, that ß-endorphin injection into the ventrolateral PAG lowers arterial pressure and heart rate, raising the possibility that ß-endorphin release within the PAG may trigger hemorrhagic hypotension. Bilateral naloxone injection into these same PAG sites attenuated the fall in arterial pressure produced by hemorrhage. These preliminary data support the hypothesis that the PAG plays an important role in hemorrhagic shock and provide a strong rationale for testing whether Gly-Gln acts in the PAG.

3. Develop a centrally active, metabolically stable Gly-Gln analog. During the present project period, we initiated collaborative studies with Dr. Ade Adejare, a medicinal chemist in the UMKC Division of of Pharmaceutical Sciences, to develop Gly-Gln analogs. The initial goal will be to investigate the Gly-Gln pharmacophore by using molecular modeling and NMR analysis. These data will subsequently be used to develop non-peptide Gly-Gln analogs that are resistant to peptidase hydrolysis and exhibit high affinity for [^{3}H]Gly-Gln binding sites.

PUBLICATIONS, ABSTRACTS, TECHNICAL REPORTS, PATENTS, AND AWARDS (12 months):

1. Millington WR (1998) Opioid peptides in the pathophysiology and treatment of hemorrhage. Abstract. Conference on Resuscitation Fluid Design and Resuscitation Protocols for Combat Casualties. National Academy of Sciences.

2. DelCampo L, Rapacon-Baker M, Resch GE, Evec A, Fibuch EE, Porter MD, Millington WR (1999) Cardiovascular effects of ß-endorphin in the midbrain periaqueductal gray region. Abstract. Midwest Anesthesiology Residents Conference.

3. Buyukbingol E, Caira MR, Millington WR, Adejare A (1999) Conformational studies of Gly-Gln and its analogs. Abstract. American Chemical Society.

ANNUAL REPORT QUESTIONNAIRE

1 June 1998 – 31 May 1999

GRANT TITLE: Control of Hemorrhagic Hypotension with Gly-Gln, a Non-Opioid ß-Endorphin Peptide

PRINCIPAL INVESTIGATOR: William R. Millington, Ph.D.

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YOUR WEB SITE URL: None

GRANT NUMBER: N00014-98-1-0249

AWARD PERIOD: January 1, 1998 - December 31, 2000

ONR PROGRAM OFFICER: Jeannine A. Majde, Ph.D.

TECHNOLOGY TRANSFER: We plan to develop Gly-Gln analogs with improved potency and reduced susceptibility to proteolytic degradation. Once we have produced and tested a prototypic compound, the appropriate data will be submitted, as a preliminary disclosure application, to the University of Missouri Patent Office. If approved, the Patent Office will seek corporate sponsors for a formal patent application. In the longer term, we plan to pursue two additional objectives which may lead to technology transfer. First, to develop a Gly-Gln analogs which, unlike Gly-Gln itself, are capable of permeating biological membranes and will be centrally active following oral or intravenous administration. This objective will be met by modifying the structure of cyclo(Gly-Gln), a cyclic Gly-Gln analog which, like other cyclic dipeptides, is centrally active following peripheral administration. A second, albeit more difficult objective, is to develop selective Gly-Gln receptor antagonists. Both classes of compounds may lead to additional opportunities for technology transfer to the private sector.

ONR DATABASE STATISTICS:

Number of Papers Published in Refereed Journals Supported by ONR 0

Number of Books or Chapters Published Supported by ONR 0

Number of Technical Reports and Non-Refereed Papers Supported by ONR 0

Number of Patents Issued: 0

Number of Patents Pending: 0

Number of Presentations: 3

Number of Degrees Granted: 0

Number of Principal Investigators (PI)/co-PI (Total): 1
- PI/co-PI Women: 0
- PI/co-PI Minority: 0

Number of Associate Investigators (AI) (Total): 1
- AI Women: 0
- AI Minority: 0

Provide names, addresses, phone numbers, fax numbers, and E-mail addresses of Co-PI and AI, if the information provided in your research proposal requires updating. NA

Post Doctoral Students Supported (Total): 1
- Post Doctoral Women: 1
- Post Doctoral Minority: 0

Number of Doctoral Students Supported (Total): 0
- Doctoral Women: ____
- Doctoral Minority: ____

Number of Masters Students Supported (Total): 0
- Masters Women: ____
- Masters Minority: ____

Number of Undergraduate Students Supported (Total): 2
- Undergraduate Women: 1
- Undergraduate Minority: 0

Other Sponsored Work:

a) Title: Regulation of Sympathoadrenal Activity by Proopiomelanocortin Neurons
Sponsor: North Atlantic Treaty Organization #SA.5-2-05(CRG.960024)276/96/JARC-501
Annual Direct Costs: $6,410
Percent Effort: 1996-1999
PI Involvment: PI (10% Effort) This is a NATO collaborative grant with Dr. Ismail Ulus, Department of Pharmacology, Uludag University. Bursa, Turkey, to study regulation of the sympathetic nervous system. The NATO grant funds travel expenses only.

b) Title: Inhibition of alcohol and morphine self-administration by non-opioid ß-endorphin peptides
Sponsor: Sarah Morrison Bequest
Annual Direct Costs: $24,389
Percent Effort: 1999 - 2001
PI Involvement: Co-PI (10% Effort). The specific aims are: (1) To determine if Gly-Gln [ß-endorphin(30-31)] or ß-endorphin(1-27) administration inhibits alcohol consumption by rats selectively bred for high alcohol drinking; (2) To test whether Gly-Gln or ß-endorphin-1-27 inhibit alcohol-induced dopamine release in the nucleus accumbens; (3) To determine if Gly-Gln or ß-endorphin(1-27) inhibit intravenous morphine self-administration.

Foreign Collaborations:
Investigator: Dr. Ismail H. Ulus
Institution: Department of Pharmacology, Uludag University School of Medicine
Location: Bursa, Turkey
Objective: To investigate the central regulation of the sympathetic nervous system.

PROJECT HIGHLIGHT

1 June 1998 – 31 May 1999

NAME: William R. Millington, Ph.D.

INSTITUTION: University of Missouri-Kansas City

PROJECT HIGHLIGHT:

The Glycyl-Glutamine Receptor: Glycyl-glutamine (ß-endorphin$_{30-31}$) is an endogenous peptide synthesized from the opioid peptide, ß-endorphin. Gly-Gln is a major product of ß-endorphin processing in brain regions that regulate cardiovascular function. Consistent with its localization, Gly-Gln administration to rats prevents the precipitous hypotension induced by severe blood loss and inhibits the hypotension and respiratory depression produced by opiate drugs. Gly-Gln's mechanism of action has yet to be defined, however. Presumably, like other neuropeptides, Gly-Gln produces its central effects by activating a membrane receptor. But it is unknown whether Gly-Gln activates a specific, 'Gly-Gln receptor' or a receptor that normally mediates the effects glycine, opioid peptides or another previously identified neurotransmitter. Identifying Gly-Gln's receptor mechanism is an essential step toward developing Gly-Gln receptor agonists and antagonists. During the current reporting period, we characterized Gly-Gln binding sites in bovine brain with a radioligand binding assay using [^{3}H]Gly-Gln as a ligand.

1. Characterization of [^{3}H]Gly-Gln Binding: The objective of the initial experiments was to determined whether [^{3}H]Gly-Gln binding satisfies criteria for receptor binding. We found that [^{3}H]Gly-Gln binding displayed the following characteristics: (a) [^{3}H]Gly-Gln binding is pH (Fig. 1) and temperature (Fig. 2) dependent. Binding was ten-fold higher at 26 °C than 4 °C although it was lower at 37 °C, presumably due to proteolytic degradation of [^{3}H]Gly-Gln binding sites (control experiments showed that [^{3}H]Gly-Gln is not metabolized by brain membranes at 4, 26 or 37 °C). (b) [^{3}H]Gly-Gln binds to a membrane protein. Binding was eliminated by preincubating brain membranes with trypsin or chymotrypsin, or by heat denaturation (Fig. 3) and it was linearly correlated with membrane protein concentrations between 50 and 700 µg. (c) [^{3}H]Gly-Gln is stereospecific, to the extent that glycyl-D-glutamine failed to displace [^{3}H]Gly-Gln from bovine brain membranes (Fig. 4); glycyl-D-glutamine was also inactive in earlier studies of hemorrhagic and opioid hypotension. (d) Subcellular fractionation experiments demonstrated that [^{3}H]Gly-Gln binding sites are localized in synaptosomes but not other subcellular fractions, consistent with the conclusion that [^{3}H]Gly-Gln binding sites are localized in neuronal synapses. (e) [^{3}H]Gly-Gln binding densities are highest in the pons and medulla, consistent with the regional distribution of Gly-Gln (Table 2).

2. Kinetics: [^{3}H]Gly-Gln binding was saturable with K_d = 44 ± 9 nM and Bmax = 1.5 ± 0.3 pmol/mg protein by Scatchard analysis using ligand concentrations between 0.1 nM and 400 nM (Fig. 5). The Scatchard curve was linear, suggesting that [^{3}H]Gly-Gln binds to a single site, and the Hill coefficient was 1.2 ± 0.1, indicating a lack of binding cooperativity. The K_d calculated from association and dissociation rate constants, was 96 ± 5 nM, consistent with, although somewhat higher, than K_d value determined from Scatchard analysis. These data indicate that [^{3}H]Gly-Gln binds to a single site with a K_d in the nanomolar range.

3. Structure-Activity Studies: [^{3}H]Gly-Gln apparently binds to a unique, Gly-Gln binding site, rather than a previously identified receptor, because it was not displaced by ligands for glycine, excitatory amino acid or opioid receptors. Conversely, Gly-Gln was inactive in [^{3}H]glycine or [^{3}H]naloxone binding assays, again indicating that it lacks affinity for glycine or opioid receptors; NIMH NovaScreen analysis confirmed that Gly-Gln lacks affinity for a wide range of standard receptor, transport and enzyme binding assays. [^{3}H]Gly-Gln was also displaced by dipeptides structurally related to Gly-Gln, albeit with substantially lower affinity than for Gly-Gln itself (Table 3). Interestingly, none of the dipeptides tested displayed an affinity for [^{3}H]Gly-Gln binding sites greater than that of Gly-Gln.

4. Summary: These data fulfill two objectives. First, the data indicate that [^{3}H]Gly-Gln binding sites satisfy many of the criteria for a neurotransmitter receptor. [^{3}H]Gly-Gln binds to a membrane protein with the expected pH optimum, temperature, time and protein dependence. Furthermore, [^{3}H]Gly-Gln is localized subcellularly in synaptosomes, as one would predict for a neurotransmitter receptor, and found regionally in highest density in the pons and medulla, regions in which ß-endorphin is almost entirely converted to Gly-Gln and related peptides. [^{3}H]Gly-Gln binding is saturable and displaceable with K_d of 44 $\pm$ 9 nM, a relatively low affinity, but well within the range of small molecule neurotransmitter receptor binding affinities. Hence, based on binding studies alone, [^{3}H]Gly-Gln apparently labels a protein binding site with characteristics consistent with those of a neurotransmitter receptor.

Secondly, the data support the hypothesis that [^{3}H]Gly-Gln binds to a unique, Gly-Gln binding site, rather than a previously identified opioid, glycine or other receptor. This conclusion is based on NovaScreen analysis showing that Gly-Gln was inactive in seventy-five receptor, transporter and enzyme binding assays and our own data showing that Gly-Gln does not displace [^{3}H]naloxone or [^{3}H]glycine binding. Characterization of [^{3}H]Gly-Gln binding sites supports this conclusion by demonstrating that ligands for glycine, opioid and other receptors fail to inhibit [^{3}H]Gly-Gln binding. Structure activity studies with dipeptides further demonstrated that relatively minor modifications of the Gly-Gln structure substantially reduce affinity for [^{3}H]Gly-Gln binding. Nevertheless, further study is warranted before accepting the conclusion that [^{3}H]Gly-Gln binding sites represent an authentic Gly-Gln receptor.

MAJOR PROBLEMS: (i.e., late receipt of funds, loss of personnel, technique doesn't work, etc.)

POTENTIAL PATENTABLE INVENTIONS: The finding that Gly-Gln prevents the fall in arterial pressure induced by severe hemorrhage and attenuates the hypotension and respiratory depression produced by opiate drugs raises the possibility that Gly-Gln or its derivatives may ultimately be useful clinically to treat these and related conditions. Gly-Gln is an endogenous brain peptide, however, and as such, it is not patentable. A long-term goal of our research is to design and synthesize novel Gly-Gln analogs. The specific objective of this effort will be to discover compounds that are metabolically stable and capable of permeating biological membranes, including the blood-brain barrier. Toward this end, we recently initiated collaborative experiments designed to characterize the Gly-Gln pharmacophore and, based on these data, synthesize potent and selective Gly-Gln analogs. These compounds may constitute potential patentable inventions.

Table 1. Subcellular distribution of [^{3}H]Gly-Gln binding sites in bovine brainstem.

Subcellular Fraction	[^{3}H]Gly-Gln Bound (pmol/mg protein)
Synaptosomal	0.88 ± 0.05
Mitochondrial	0.10 ± 0.03
Endoplasmic Reticulum/Golgi	ND
Myelin	ND

Subcellular fractions from bovine brainstem were prepared by sucrose density centrifugation and incubated with 20 nM [^{3}H]Gly-Gln for 60 min. Non-spedific binding was determined with 10 μM Gly-Gln. Data represent the mean ± SE (n = 3). ND = Not Detectable.

Table 2. Regional distribution of [^{3}H]Gly-Gln binding sites in bovine brain.

Region	^{3}H]Gly-Gln Bound (fmol/mg protein)
Medulla	135 ± 34
Pons	167 ± 40
Midbrain	55 ± 13
Hypothalamus	68 ± 15
Thalamus	65 ± 9
Hippocampus	45 ± 13
Caudate	62 ± 8
Cortex	87 ± 18
Cerebellum	34 ± 12

Bovine brain membranes were prepared from the indicated brain regions and incubated with 20 nM [^{3}H]Gly-Gln for 60 min in the presence or absence of 10 μM Gly-Gln. Data represent the mean ± SE (n = 4)

Table 3. Dipeptide inhibitors of [^{3}H]Gly-Gln binding.

Inhibitor	K_i (μM)
Glycyl-L-Glutamine	0.2 ± 0.1
Seryl-Glycine	0.6 ± 0.2
Glycyl-L-Asparagine	1.6 ± 0.3
Glutaminyl-Glycine	1.9 ± 0.1
Leucyl-Glycine	2.4 ± 0.2
Glycyl-L-Leucine	3.8 ± 0.3
Glycyl-L-Glutamate	5.1 ± 1.6
Glycyl-Glycine	8.4 ± 1.1
Seryl-L-Leucine	24.6 ± 3.1
Glycyl-D-Glutamine	> 100

Bovine brain membranes were incubated for 60 min with 10 nM [^{3}H]Gly-Gln and varying concentrations (1 nM - 10 mM) of the indicated inhibitor. Data represent the mean ± SE. (n=4).

FIGURE LEGENDS

Figure 1. The pH optimum of [^{3}H]Gly-Gln binding. Bovine brain membranes were incubated for 60 min with [^{3}H]Gly-Gln (20 nM) at the indicated pH. Non-specific binding was determined with 10 μM Gly-Gln (n = 3). Data represent the mean ± SEM of four experiments.

Figure 2. Temperature dependence of [^{3}H]Gly-Gln binding. Bovine brain membranes were incubated with [^{3}H]Gly-Gln (20 nM) at the indicated temperature. Data represent the mean ± SEM of three experiments.

Figure 3. Protease incubation and heat denaturation abolish [^{3}H]Gly-Gln binding. Bovine brain membranes were pre-incubated with either trypsin (1 mg/ml), α-chymotrypsin (1 mg/ml) or heated at 60 °C for 40 min, then incubated with [^{3}H]Gly-Gln (20 nM) for 60 min. Non-specific binding was determined with 10 μM Gly-Gln (n = 3).

Figure 4. [^{3}H]Gly-Gln binding is stereospecific. Bovine brain membranes were incubated with [^{3}H]Gly-Gln (20 nM) and the indicated concentration of Gly-Gln (circles) or glycyl-D-glutamine (squares) for 60 min (n = 4).

Figure 5. Saturation curve and Scatchard analysis (insert) of [^{3}H]Gly-Gln equilibrium binding to bovine brain membranes. Bovine brain membranes were incubated with the indicated concentration of [^{3}H]Gly-Gln. Non-specific binding was estimated with 10 μM Gly-Gln. K_d = 44 ± 33 nM; B_{max} = 1.51 ± 0.34 pmol/mg protein (n = 14).

FIGURE 1

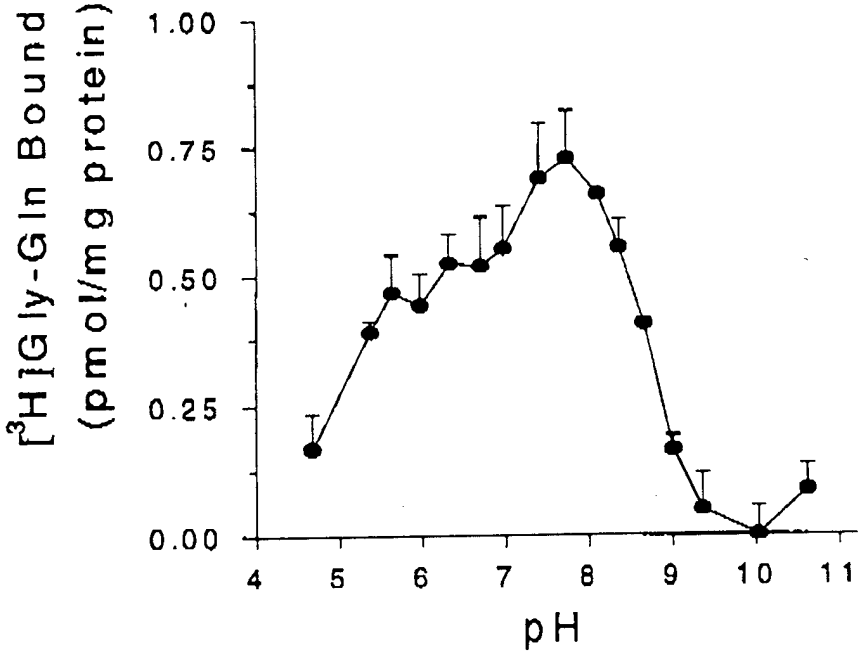

FIGURE 2

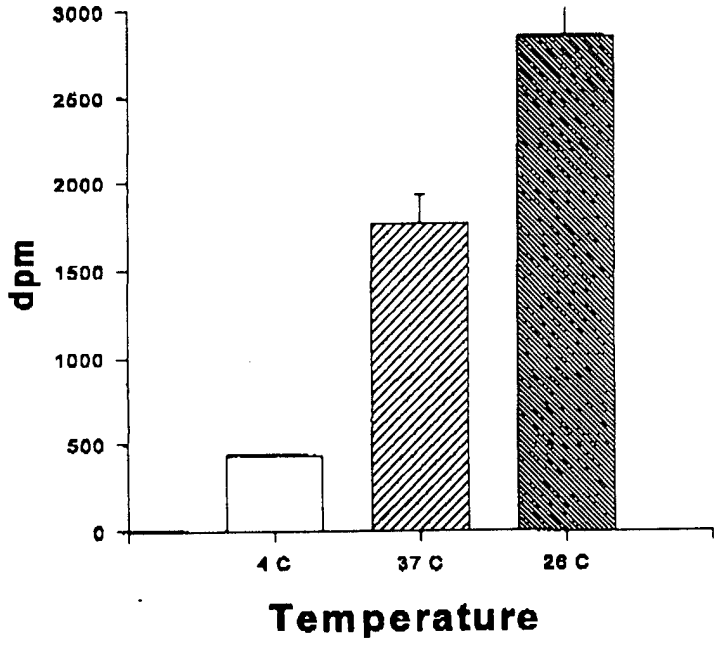

FIGURE 3

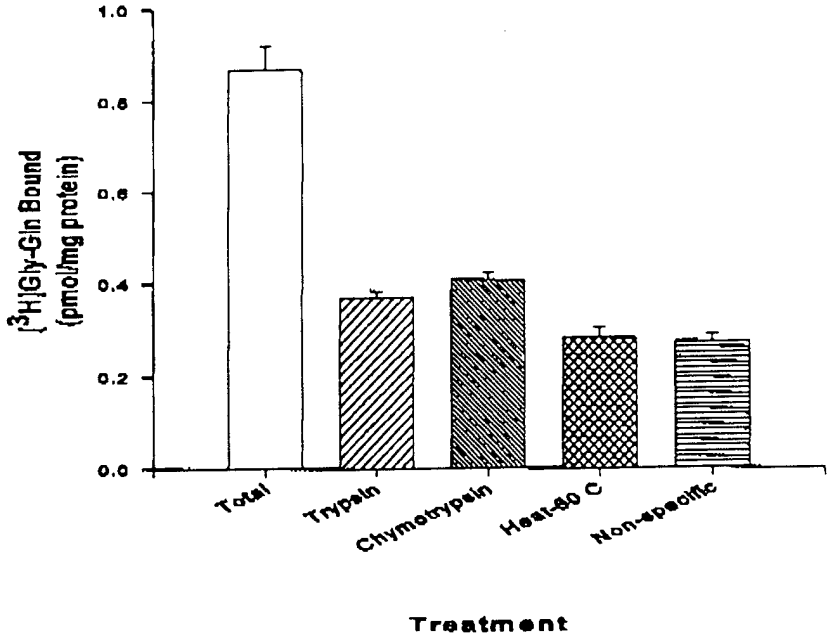

FIGURE 4